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Iron Deficiency during Early Development in the Rat: Behavioral and Physiological Consequences[1]

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WEINBERG, J., P. R. DALLMAN AND S. LEVINE. *Iron deficiency during early development in the rat: Behavioral and physiological consequences.* PHARMAC. BIOCHEM. BEHAV. 12(4)493–502, 1980.—An iron deficient diet regimen from birth through 28 to 30 days of age produced animals with decreased levels of brain non-heme iron as well as decreased brain weight and hematocrit. Weight gain was also somewhat slower. In contrast, brain levels of cytochrome c were not reduced. During subsequent testing iron deficient animals were less responsive than controls in a mildly aversive novel situation (the open field), and ambulated less in an exploratory task (the hole-board). Iron deficient males also exhibited longer response latencies when first exposed to the passive avoidance platform, but following shock, iron deficient animals of both sexes exhibited longer reentry latencies. Measurement of plasma levels of corticosterone indicated that although iron deficient animals had elevated basal levels of corticoids, they exhibited a smaller stress increment than controls when exposed to the combined stress of ether and cardiac puncture. Data from the three behavioral tasks taken together with the pituitary-adrenal response to ether and cardiac puncture suggest that iron deficiency may reduce an animal's general responsiveness to environmental stimuli.

Iron deficiency Brain non-heme iron Brain cytochrome c Open field Hole-board
Passive avoidance Pituitary-adrenal system Rat

IRON lack is considered to be the most prevalent nutritional deficiency in the U.S. [31]. It crosses all socioeconomic lines but is most common among the poor, and in infants, children, and women during the childbearing years [49]. Despite its widespread occurrence, however, there is little consensus as to the behavioral or functional consequences of iron deficiency. First, even severely anemic patients are sometimes asymptomatic [7,48]. In fact, most cases of iron deficiency and/or anemia are detected through screening of high risk groups rather than through self-reports of symptoms. Second, symptoms, when they do occur, are often vague (e.g., fatigue, irritability, loss of appetite), and the number and severity of symptoms do not appear to correlate with the degree of anemia [19]. Third, in studies where behavioral changes have been reported, the results are often inconclusive or difficult to interpret. Anemic children have been reported to have a higher incidence of "soft" neurologic signs such as inattentiveness and hyperactivity [10], to score lower on tests of mental ability [30,36] or on achievement tests [39], and to be less attentive, less able to focus on a task, and more restless and disruptive in a classroom situation than nonanemic children [25, 39, 40]. However, many of these studies have flaws in design. Frequently there is no information on statistical analyses, methods of testing, or definition of tasks. Groups are often not matched on social or environmental variables, and appropriate controls are often omitted. Further, it is often unclear whether the results obtained are due to iron deficiency, or are instead due to a general state of malnutrition and/or a deficient social and intellectual environment (see [27] for review).

The relationship between anemia and physical activity appears to be more firmly established. Several studies on rodents indicate that iron deficiency reduces both voluntary activity and ability to withstand forced exercise [18,21]. A relationship between anemia and reduced physical endurance in humans has also been reported [4, 6, 11, 37]. However, it is not known whether milder degrees of anemia affect the non-maximum work performance typical of most occupations.

Because of the limitations of behavioral studies in humans, some investigators have begun to develop animal models in which environmental and nutritional variables can be more carefully controlled [14, 18, 21]. In one series of studies [14] lactating females with 10 day old litters were given either an iron deficient or an otherwise identical con-

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trol diet. Pups were continued on their respective diets from weaning (Day 21) until either 28 or 48 days of age. At both ages iron deficient animals were found to be significantly below controls in brain total non-heme and ferritin iron, as well as in hematocrit, and liver non-heme and ferritin iron. Body weight was also below control values at both ages, but was decreased to a greater extent in the older group. All iron deficient animals were then given 5 mg of iron (as iron dextran) IM, and given the control diet regimen. Under similar conditions, it was observed that hemoglobin and liver non-heme iron, after approximately 1 week [13] returned to control values. However, the deficiency of total non-heme iron and of ferritin iron in the brain persisted.

These conditions under which long-lasting or irreversible deficiencies in brain iron content occurred appeared to provide an interesting animal model for studying the effects of iron deficiency on behavior. Twenty-eight day old animals were chosen for study because iron deficiency did not result in further depletion of brain non-heme iron after this age. Furthermore, the relatively brief duration of iron deprivation has just begun to result in a decreased rate of growth at this age; continuation of the iron deficiency regimen results in more substantial secondary depression of food intake and weight gain.

Certain procedures involving handling, housing, cleaning and culling litters had to be modified if the pups raised on these diet regimens were to be used for subsequent behavioral testing. Therefore the first experiment was undertaken to modify the model established by Dallman *et al.* [14] in order to establish conditions suitable for behavioral studies, and determine the effects of these procedural changes on the biochemical parameters established previously.

EXPERIMENT 1

In this experiment we examined the effects of varying periods of perinatal iron deficiency.

METHOD

Animals

Twenty (primiparous) pregnant females were obtained from Simonsen Laboratories, Gilroy, CA. Animals were housed individually in clear plastic cages (28×48×24 cm), with wood shavings as bedding, in a colony room with controlled temperature and humidity, and maintained on a 12 hr light/12 hr dark cycle with lights on at 0830 hr.

Diet Conditions

Animals were assigned to one of three treatment groups as follows:

Group A. Females on Day 18 of gestation were provided either an iron-supplemented control diet (Teklad Normal Protein Test Diet Cat. No. 170590) or the same diet with omission of the iron salt (Teklad Normal Protein Test Diet with Iron Deleted) and distilled drinking water (n=4 per condition).

Group B. Females were provided the diets on the day of birth; four females received the iron deficient diet and 4 received the control diet.

Group C. Females were provided the diets when pups were 10 days of age; two females received the control diet and two females received the iron deficient diet.

At birth all litters were culled to 8 (4 males, 4 females). Because pups are coprophagic and thus obtain some iron from the dams, we also assessed the effects of various cage cleaning schedules. This was done in order to establish a schedule which would minimize handling of the litters and still maintain adequate levels of iron deficiency. Thus, for all groups, cages were cleaned on Days 10, 14 and 18 postpartum. Further, for Groups A and B, half the litters in each diet condition were cleaned on Days 4 and 7 postpartum while the remaining litters were left undisturbed until Day 10.

All litters were weaned on Day 21; pups were housed 2 per cage in 28×48×24 cm plastic cages and all were maintained on their respective diets until 28 days of age.

Measurements

On Day 28, 2 animals from each of the 8 litters in Groups A and B, and 2 from each of the 4 litters in Group C were selected for testing. The following measurements were taken: (1) Body weight; (2) Hematocrit: Hematocrits were determined by centrifugation on blood collected from the tail in heparinized glass capillary tubes; (3) Brain non-heme iron: Animals were injected with pentobarbital anesthesia and perfused with heparinized isotonic saline via the left ventricle of the heart. The brain was then removed, weighed, and whole brain non-heme iron was determined by the method of Weinfeld [42]. This procedure is described more completely in Dallman *et al.* [14]; (4) Brain cytochrome c: An additional 6 animals were selected from each diet condition in Group B; brains from 2 animals were pooled for each analysis. Cytochrome c was measured spectrophotometrically [46].

RESULTS AND DISCUSSION

Cage cleaning prior to Day 10 had no effect on any measure; therefore scores for "cleaned" and "not cleaned" groups were combined for the analysis.

For each treatment age (A,B,C) separate 2 (Diet)×2 (Sex) analyses of variance were performed on the measures of body weight, brain weight, hematocrit and brain non-heme iron. Within each treatment age group it was found that iron deficient animals were significantly below controls on all of these measures (see Tables 1 and 2). In addition, significant sex differences occurred on several of the measures. Males were generally heavier than females ($ps<0.01$ in Groups B and C), and males in all 3 groups had significantly higher brain weights than females ($ps<0.05$ in Groups A and B; $p<0.01$ in Group C). However, although significant, this brain weight difference was of very small magnitude (approximately 3 to 5%), and should be considered within the context of the males' higher body weights. In addition, females in Group A had significantly higher levels of brain non-heme iron than males ($p<0.01$). This suggests that males may be slightly more vulnerable to the effects of iron deficiency, perhaps as a consequence of their more rapid rate of growth.

Iron deficient and control animals in the "not cleaned" condition were also compared across the three treatment ages in order to determine whether age of starting the diet significantly affected any of the parameters. A 3(Age)×2 (Diet)×2 (Sex) analysis of variance was carried out on measures of body weight, brain weight, hematrocrit, and brain non-heme iron. There was no effect of treatment age on brain weight. However, significant effects were observed on the other three measures. Animals started on the dietary regimen at 10 days of age weighed more than animals started on regimens at either 18 days gestation or at birth ($ps<0.05$) and

TABLE 1

SUMMARY OF RESULTS FOR BRAIN AND BODY PARAMETERS AT 28 DAYS OF AGE*

Treatment age	Diet	Sex	Body weight	Brain weight	Hematocrit	μg Fe/g brain
A. 18 days gestation	−I	♂ (8)	71.9 ± 3.9†	1.51 ± 0.02	12.1 ± 0.7	4.0 ± 0.1
		♀ (8)	67.3 ± 6.0	1.44 ± 0.03	14.7 ± 1.3	4.3 ± 0.2
	C	♂ (9)	95.8 ± 2.6	1.63 ± 0.02	36.0 ± 0.8	6.1 ± 0.2
		♀ (7)	92.6 ± 2.2	1.59 ± 0.01	36.4 ± 1.1	7.1 ± 0.1
B. Birth	−I	♂ (8)	71.6 ± 2.0	1.54 ± 0.02	12.3 ± 0.5	4.3 ± 0.2
		♀ (8)	61.8 ± 1.2	1.50 ± 0.03	12.1 ± 0.5	3.9 ± 0.2
	C	♂ (8)	101.3 ± 3.4	1.60 ± 0.02	37.9 ± 0.9	6.3 ± 0.2
		♀ (8)	88.3 ± 1.5	1.53 ± 0.03	38.7 ± 1.0	6.3 ± 0.3
C. Pups 10 days of age	−I	♂ (4)	90.5 ± 0.6	1.60 ± 0.01	14.6 ± 0.6	4.8 ± 0.5
		♀ (4)	67.3 ± 8.7	1.47 ± 0.04	17.7 ± 0.3	4.6 ± 0.3
		♂ (4)	103.3 ± 3.5	1.63 ± 0.03	38.4 ± 1.0	6.1 ± 0.1
		♀ (4)	94.5 ± 1.8	1.59 ± 0.02	39.0 ± 1.5	6.4 ± 0.2

*Includes "cleaned" and "not cleaned" groups.
†Mean ± SEM.
( ) Number of animals.

TABLE 2

SUMMARY OF ANALYSES OF VARIANCE [2 (DIET) × 2 (SEX) FOR EACH TREATMENT AGE]

Treatment age	Main effect of diet for:	F	p
A	Body wt.	F(1,28)= 38.9	<0.0001
	Brain wt.	F(1,28)= 34.2	<0.0001
	Hematocrit	F(1,28)= 535.9	<0.0001
	Brain iron	F(1,28)= 187.6	<0.0001
B	Body wt.	F(1,28)= 164.2	<0.0001
	Brain wt.	F(1,28)= 3.2	<0.08
	Hematocrit	F(1,28)=1105.2	<0.0001
	Brain iron	F(1,28)= 107.5	<0.0001
C	Body wt.	F(1,12)= 17.6	<0.01
	Brain wt.	F(1,12)= 8.3	<0.02
	Hematocrit	F(1,12)= 549.6	<0.001
	Brain iron	F(1,12)= 23.9	<0.001

these latter two groups did not differ from each other. In addition, a significant Age×Diet interaction, $F(2,36)=7.5$, $p<0.01$, indicated that although treatment age had no consistent effect on hematocrits of control animals, hematocrits of animals provided the iron deficient diet at 10 days of age were significantly higher than those of animals provided the diet at 18 days gestation or at birth ($ps<0.01$). The age of starting on the diet also affected levels of brain non-heme iron, $F(2,36)=6.9$, $p<0.01$. Brain iron levels were higher in animals provided the iron deficient diet at 10 days of age than in animals provided the same diet at birth or at 18 days gestation ($ps<0.01$). Also, control animals provided the diet at 18 days gestation had slightly higher brain non-heme iron levels than animals in the other two treatment ages ($ps<0.05$).

Finally, measurement of brain cytochrome c indicated no reduction in iron deficient animals compared to control animals (mean=3.24 ± 0.03 n mole/g brain for iron deficient and mean=3.10 ± 0.03 n mole/g brain for control animals; $t_4=3.4$, $p<0.05$). Indeed, levels of brain cytochrome c were slightly higher in iron deficient animals.

These results indicate that placing animals on the iron deficient regimen at 18 days gestation or on the day of birth produces more severe iron deficiency with respect to hematocrit and brain non-heme iron than if treatment is begun at 10 days of age.

In light of these data the following procedures were established for all subsequent experiments: Pregnant females were obtained from Simonsen Laboratories, Gilroy, CA. The females were housed individually in plastic cages (28×48×24 cm) with wood shavings as bedding, and a grid top which held a water bottle and food pellets. The colony

room was maintained with controlled temperature and humidity, on a 12 hr light/12 hr dark cycle with lights on at 0800 hr. On the day of birth all litters were culled to 8 (4 males, 4 females) and females were placed on their respective diets. Cage cleaning occurred on Days 10, 14 and 18. After weaning on Day 21 pups were housed 2 per cage with a littermate of the same sex, and were maintained on their respective diets until 28 to 32 days of age, depending upon when testing was completed. Following testing all animals were weighed, and several animals from each condition were randomly selected for determination of hematocrits. In all subsequent studies it was established that weights and hematocrits conformed to the parameters established in this experiment.

EXPERIMENT 2

In Experiment 1 we demonstrated significant effects of early iron deficiency on hematocrit, brain non-heme iron, brain weight and body weight. In this experiment we began our investigation of the effects of early iron deficiency on behavior. Alterations of an infant animal's nutritional status is one manipulation which has been shown to affect many aspects of behavioral and physiological development (e.g., [32, 35, 44]). However, aside from studies on forced exercise performance (e.g., [18,21]) few studies have been undertaken to determine the behavioral or functional consequences of iron deficiency. In this experiment we investigated the response of iron deficient animals to a novel environment. The task chosen was the open field.

METHOD

Animals

Fourteen pregnant females were obtained for this experiment. One female was excluded from the study because only 5 viable pups were delivered. Of the remaining females, 7 were provided the iron deficient diet regimen and 6 were provided the control diet regimen on the day they gave birth. All conditions of housing, culling, weaning, and post-weaning treatment were as described at the end of Experiment 1. At 29 days of age, 11 males and 11 females were selected from each diet condition, with the restriction that no more than 3 animals (either 2 males and 1 female or 1 male and 2 females) were selected from any one litter.

Apparatus

A circular open field patterned after Broadhurst [8] was used. It consisted of an arena 84 cm in diameter with 33 cm walls, all of white painted plywood. The floor was divided by black lines into three concentric circles with sections of radii that divided the outer circle into 12 sectors and the middle circle into six sectors. All testing was done in a darkened room. The field was brightly and evenly illuminated by two 150 W flood lamps in 23-cm aluminum reflectors suspended 91 cm above the field. A white masking noise of approximately 75 dB was delivered by a 9-cm speaker, also suspended 91 cm above the field.

Procedure

Animals were tested for 3-min sessions daily for 2 consecutive days. Order of testing was balanced across each session. At the start of each trial the animal was placed in the center of the field. Measures recorded for each session included (1) Ambulation: number of sectors entered with all 4 paws; (2) Rearing: 2 front paws lifted off the floor; (3) Freezing: standing immobile for more than 10 sec; (4) Defecation. Ambulation and rearing scores were tallied on a mechanical counter, and duration of freezing was timed on a stop watch. All ratings were made by an experienced observer. Between trials the field was wiped clean and then washed down with a 50% EtOH solution to neutralize olfactory cues. Each behavioral measure was analyzed by a 2 (Diet)×2 (Sex)×2 (Days) analysis of variance, with the Days factor treated as a repeated measure. Post hoc testing was by tests of simple main effects [47].

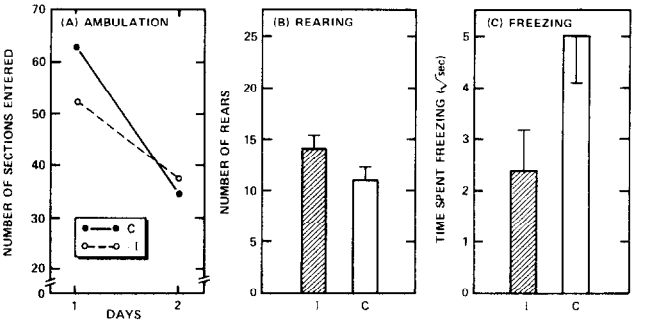

FIG. 1. Responses during open field testing in iron deficient (-I) and control (C) animals. (Mean+SEM in panels B and C).

RESULTS AND DISCUSSION

Ambulation

All groups ambulated less on Day 2 than on Day 1 of testing, $F(1,40)=51.6$, $p<0.0001$. In addition, the Diet×Days interaction was significant, $F(1,40)=4.82$, $p<0.05$. There was a tendency for control animals to ambulate more than iron deficient animals on Day 1 of testing, $p<0.10$, but the groups were similar by Day 2 (Fig. 1A).

Rearing

Although all animals showed a decrease in rearing over the 2 days of testing, $F(1,40)=58.6$, $p<0.0001$, iron deficient animals reared more than control animals, $F(1,40)=4.3$, $p<0.05$ (Fig. 1B).

Freezing

Freezing rarely occurred on Day 1 and therefore only the Day 2 scores were analyzed. Because of the high variability of scores, a square root transformation was performed on the data. Iron deficient animals showed significantly less freezing than controls, $F(1,40)=4.7$, $p<0.05$ (Fig. 1C).

Defecation

Groups did not differ in amount of defecation in this task.

The open field has been widely used to test responses of animals, particularly rodents, to a mildly aversive novel environment. However, interpretations of the meaning of open field behavior vary considerably from study to study [5,38]. Because the measures of motor activity, i.e. ambulation and rearing, tend to be positively correlated with each other, and negatively correlated with freezing, some investigators use open field motor activity scores as indicative of exploration in a novel environment [9,23]. However, it has been shown that ambulation is a factorially complex varia-

ble, measuring not only exploration but also emotional reactivity [43]. It appears that high ambulation scores on Day 1 of testing reflect high emotionality and may in fact be motivated by attempts to escape, while high ambulation scores on subsequent test days reflect reduced emotionality and the tendency to explore. Thus the open field appears to provide a measure of emotionality or fearfulness in addition to a measure of exploration [38]. Furthermore, it appears that one must examine the pattern of scores over 2 or more days of testing rather than scores obtained on a single test day in order to assess the meaning of open field performance.

In this experiment we compared the behavior of iron deficient and control animals over 2 days of testing in an open field. We observed that iron deficient rats ambulated less than controls on Day 1 of testing, and in addition displayed more rearing and less freezing. These results were unanticipated. Because iron deficiency has such marked effects on both brain and body measures, it was surprising to find that iron deficient subjects displayed a pattern of response indicative of increased exploration and reduced emotionality [43]. The next experiment was designed to further investigate these findings.

TABLE 3
HOLE-BOARD SCORES

A) Head-dipping

		Day 1	Day 2
−I	♂	2.8 ± 0.7	0.6 ± 0.3
	♀	3.4 ± 0.9	1.6 ± 0.6
C	♂	1.9 ± 0.4	0.3 ± 0.2
	♀	3.5 ± 0.6	0.7 ± 0.3

B) Ambulation

	Day 1	Day 2
−I	24.6 ± 0.7	11.7 ± 1.2
C	29.3 ± 1.7	11.9 ± 1.9

EXPERIMENT 3

Because locomotor behavior (i.e., ambulation) is a factorially complex variable [43] it is difficult to separate exploratory behavior from emotional reactivity in a task such as the open field. The open field can be considered a "forced exploration" procedure; that is, the animal is placed into a novel environment from which there is no escape. It has been suggested [12] that a "choice" task may provide a better measure of exploration; that is, the animal is placed into a novel environment, but the exploratory response is a behavior which is relatively independent of ambulation, or at least allows the animals to choose between 2 or more different areas within that environment (e.g., [16, 17, 20, 26]).

The present experiment utilized the hole-board [20] in order to allow a better separation of activity, exploration, and emotional reactivity than was possible in Experiment 2. The primary dependent variable in this task is head-dipping, a response which is relatively independent of ambulation since the rat can freely ambulate without head-dipping or can stand in place and repeatedly head-dip. Previous data have demonstrated that animals which differ in emotionality also differ in amount of head-dipping in the hole-board; i.e., less emotional animals show more head-dipping [41]. We hypothesized that if iron deficient animals are less emotionally reactive and tend to explore more than controls, as suggested by the open field data, then iron deficient animals should also head-dip significantly more than controls in the hole-board.

METHOD

Animals

The same 15 litters which supplied animals for Experiment 2 provided subjects for this study. Eleven males and 11 females were randomly selected from each diet condition for testing at 27 to 28 days of age. As in Experiment 2, no more than 3 animals were selected from any one litter.

Apparatus

The apparatus was a modified rat hole-board [20]: a gray wooden box with a floor 66×66 cm and walls 35 cm high. The floor was marked off into four equal quadrants, and in the floor were four equally spaced holes, each 3 cm in diameter. The walls of the apparatus extend below the level of the floor, which was thus raised to a height of 11 cm so that objects could be placed under each hole. The objects were matches, a cork, a rubber stopper, and a ball of cotton, each standing in the mouth of a 50-ml Erlenmeyer flask. The area under the floor was illuminated by two 10 W bulbs. Testing was done in a darkened room under red light, with white masking noise of approximately 70 dB.

Procedure

All testing was conducted between 0900 and 1300 hr. Testing order was balanced across each session. Each animal was tested for a 5-min trial each day for 2 consecutive days. Between trials the apparatus was wiped clean and then washed down with a 50% EtOH solution to neutralize olfactory cues. Behavioral measures recorded were (1) head-dipping (scored if both eyes disappeared into the hole); (2) total duration of head-dipping (recorded in seconds); (3) ambulation (defined as number of quadrants entered with all 4 feet). These measures were recorded by an experienced observer using a stop watch and a check sheet. Data for each measure were analyzed in separate 2(Diet)×2(Sex)×2(Days) analyses of variance, with the Days factor treated as a repeated measure. Post hoc testing was carried out by tests of simple main effects [47].

RESULTS AND DISCUSSION

Head-Dipping

Females head-dipped significantly more than males, $F(1,40)=5.18$, $p<0.05$, and all groups head-dipped less on Day 2 than they did on Day 1 of testing, $F(1,40)=25.1$, $p<0.0001$. There was no effect of diet (Table 3A).

Duration of Head-Dipping

There was a trend for females to head-dip for longer durations than males, $F(1,40)=3.14$, $p<0.08$; and all groups head-dipped for shorter durations on Day 2 than they did on

Day 1, $F(1,40)=26.97$, $p<0.0001$. Again, there was no effect of diet condition.

Ambulation

All groups ambulated more on Day 1 than on Day 2 of testing, $F(1,40)=226.05$, $p<0.0001$. In addition, there was a significant Diet×Days interaction, $F(1,40)=5.07$, $p<0.05$. Post hoc testing indicated that control animals ambulated more than iron deficient animals on Day 1 of testing, $p<0.05$, but by Day 2 had decreased to the level of the iron deficient animals (Table 3B).

In summary, no differences were observed in head-dipping in this task. Thus, these data do not support the suggestion that iron deficient animals are less emotional and tend to explore more than controls. However, a significant difference in ambulation similar to that observed in the open field did occur, i.e. iron deficient animals showed reduced ambulation on Day 1 of testing.

One possible explanation for these results is that iron deficiency does not alter an animal's emotionality but instead alters its general responsiveness to environmental stimuli. For example, it is possible that iron deficient rats were simply less responsive to the aversive elements in the open field and therefore did not respond as much on the behavioral measures. This possibility appears to be supported, at least in part, by the data from the present experiment. In this situation, which was novel but not highly aversive, iron deficient animals ambulated less than controls on Day 1 of testing and were similar to controls in amount of head-dipping. Thus the differences between iron deficient and control animals in these two situations may not reflect emotionality differences but simply activity differences. These activity differences may indicate something about the capacity of these animals to respond to their environment. This possibility was further tested in the next experiment.

EXPERIMENT 4

This experiment was designed to determine whether differences in activity levels and in responsiveness to environmental stimuli observed in response to novel environments would also be observed in a more aversive shock-motivated situation. Furthermore we examined an aspect of behavior that could not be measured in the previous two behavioral tasks—the capacity to inhibit a response. Our task for this experiment was a step-through passive avoidance situation.

Previous data have demonstrated that passive avoidance performance is influenced by nutritional status. Thus, perinatally malnourished rats show significantly longer latencies to return to the site of shock on the first postshock trial, and hence exhibit better passive avoidance performance than controls (see [28] for review). It has been suggested that undernutrition may reduce the threshold of arousal to noxious stimuli [29], particularly shock stimuli [33]. Although iron deficient animals are not malnourished, they are significantly underweight when compared with controls (Experiment 1). This would make it difficult to separate those effects due to iron deficiency per se from those due to decreased dietary intake. Therefore, in addition to the iron deficient and control groups, a weight control group was included in this experiment. At weaning, two groups of animals were given the control diet regimen. The first group, designated the ad lib control group, was given ad lib access to the control diet until 32 days of age. Animals in the second group, designated the weight control group, were also given the control diet, but intake was restricted so that they approximated the weight gain of the iron deficient rather than the control animals.

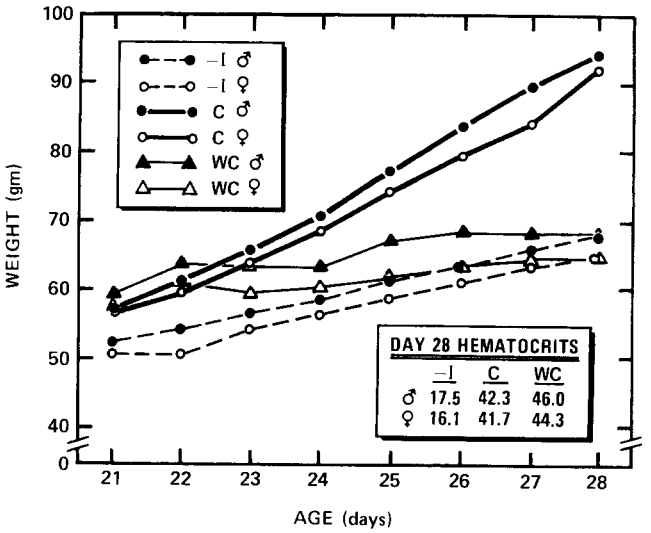

FIG. 2. Growth curve from 21 to 28 days of age in iron deficient (-I), ad lib control (C) and weight control (WC) animals.

METHOD

Animals

Sixteen pregnant females were used for this experiment. On the day of birth 5 lactating females were given the iron deficient diet and the remaining 11 were placed on the control regimen. After weaning (Day 21) pups were weighed, housed 2 per cage, and continued on their respective diets. Pups from 5 of the control diet litters were assigned to the weight control condition. Previously we had determined that restricting animals to 3 to 4 g of food per day would reduce their growth rate to approximately that of the iron deficient animals. Thus each pair of animals in the weight control condition was given 8 g of control diet on Day 21. All pups were then weighed daily. Each pair of animals in the weight control condition was given 6 to 8 g of food per day. Animals in the iron deficient and ad lib control groups were continued with ad lib access to food. We thus had 3 groups of animals: (1) iron deficient; (2) ad lib control; and (3) weight control, with weights similar to iron deficient animals, but hematocrits similar to normal animals (Fig. 2). On Day 26, 9 males and 9 females were randomly selected from each diet condition.

Apparatus

The apparatus was a modification of one described by Ader *et al.* [1]. It was a black Lucite chamber 39×39×40 cm. The floor of the box consisted of 0.3-cm rods spaced 1.4 cm apart. A 10-cm wide mesh-covered runway extended out 25 cm from the center of the front wall on a plane with the grid floor. A guillotine door separated the runway from the box. The box remained dark, while a 25 W bulb was fixed approximately 56 cm above the runway. During testing the room was dark. The box was placed with the front wall at the edge of a table so that the platform extended out over the floor.

Procedure

All testing was done between 0830 and 0100 hr. Animals

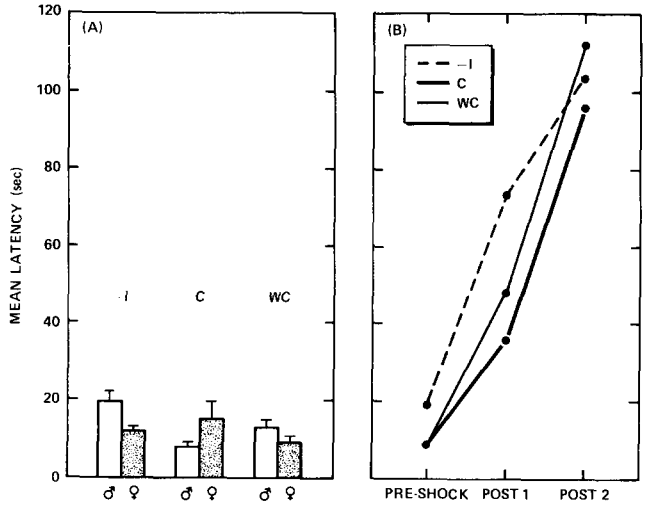

FIG. 3A. Mean latencies to enter the chamber on the Habituation Day, measured in iron deficient (-I), ad lib control (C) and weight control (WC) animals. (Mean+SEM). FIG. 3B. Mean latencies to enter the chamber on the Test Day, recorded on the trial prior to shock (PRE-SHOCK) and on the first two trials immediately following shock (POST-SHOCK 1 and POST-SHOCK 2).

were 27 to 32 days of age. Testing order was balanced within each session.

On Day 1 of testing (Habituation Day), each animal was placed into the box (with guillotine door open) and allowed to explore freely for 2 min. Animals were then removed from the chamber and immediately placed at the end of the platform facing away from the door. Latency to enter the chamber was recorded. On Day 2 (Test Day) animals were again placed on the platform, facing away from the door; latency to enter the chamber was recorded. When the rat entered, the guillotine door was immediately closed and 3 sec of 0.4 mA shock was administered. Rats were left in the chamber for an additional 7 sec and then removed to a holding pan for a 1-min intertrial interval. Animals were then placed back on the platform as before, and latency to reenter the chamber was recorded up to a maximum of 2 min. If the animal reentered within 2 min the door was closed and shock was administered as on the first trial. After 1 min in the holding pan the rat was again placed back on the platform. This procedure was repeated until the animal reached a criterion of two successive avoidances (2 min on the platform).

RESULTS AND DISCUSSION

Habituation Day

Scores for latency to enter the chamber on Day 1 of testing were analyzed by a 3 (Diet)×2 (Sex) analysis of variance. There was a significant Diet×Sex interaction, $F(2,54)=4.9$, $p<0.01$ (Fig. 3A). Post hoc tests for simple main effects [47] indicated that there was no difference in latency among females of three diet groups. However, iron deficient males showed significantly longer latencies to enter the chamber than control males, $p<0.01$, while males in the weight control condition showed intermediate latencies and were not significantly different from either of the other two groups.

Test Day

A 3(Diet)×2(Sex) analysis of variance revealed that groups did not differ on number of trials required to reach criterion (two consecutive trials of no reentry).

Latency scores for the first three trials on this test day were then subjected to a 3 (Diet)×2 (Sex)×3 (Trials) analysis of variance, with the Trials factor treated as a repeated measure. The main effect of Diet was significant, $F(2,54)=3.0$, $p<0.05$, with iron deficient animals displaying significantly longer latencies than controls, $p<0.05$, and weight controls not significantly different from either of these two groups. However, a Diet×Trials trend, $F(4,108)=2.2$, $p<0.07$, revealed that this effect was due primarily to a simple main effect of diet on the trial immediately following shock ($p<0.01$); iron deficient animals showed significantly longer reentry latencies than both ad lib controls ($p<0.01$) and weight controls ($p<0.05$) only on this trial (Fig. 3B). Groups were not different from each other either on the trial just prior to shock, or on the second trial following shock.

Passive avoidance is a conflict situation for animals. In the task employed in this experiment animals were first permitted to run from an aversive to a preferred location: the platform was narrow, brightly lit and extended out over the floor at a height of about 3 ft, while the chamber was small, dark and "safe." When placed on the platform on Day 1 of testing, all animals turned and entered the chamber. However, on Day 2 of testing animals received shock immediately upon entering. Therefore, the next exposure to the platform placed the animals in an avoidance-avoidance conflict—both the platform and the chamber were highly aversive. Under these conditions iron deficient and control animals differed in their responses. When first placed on the platform, prior to receiving any shock, all females entered the chamber with similar latencies, while iron deficient males showed significantly longer entry latencies than males in the control group. However, after receiving shock in the chamber, iron deficient animals of both sexes displayed significantly longer reentry latency than either ad lib controls or weight controls.

The increased latency to enter the chamber shown by iron deficient males during the first exposure to the platform can be viewed as support for the suggestion in Experiment 3 that iron deficiency may reduce responsiveness to environmental stimuli. However, the increased latency shown by iron deficient animals following shock appears to contradict the notion of reduced responsiveness. One possible explanation for this finding is that early iron deficiency, like early malnutrition, may permanently alter the animals' threshold of arousal to noxious stimuli such as shock. Thus a shock stimulus of a given intensity may be more noxious to iron deficient than to control animals. Data on iron deficient animals tested for threshold of response to a heat stimulus as rehabilitated adults appear to support this hypothesis. Those data indicate that rehabilitated animals show significantly shorter latencies to respond to heat than controls (unpublished). Thus it is possible that iron deficient animals may be hyperresponsive to shock because of a reduced shock or pain threshold. Furthermore these changes in responsiveness appear to be due primarily to the effects of iron deficiency, since weight control animals exhibited a response to shock which was similar to that of ad lib controls.

EXPERIMENT 5

The results of Experiment 4 indicate that while iron deficient animals may show reduced responsiveness to novel or

mildly aversive stimuli, they appear to show increased responsiveness to noxious stimuli such as shock. It was suggested that iron deficiency may reduce the threshold of arousal to noxious stimuli. Because the pituitary-adrenal system appears to provide a sensitive measure of changes in levels of arousal [24], we designed this experiment to further test this possibility by examining the animals' response to another noxious stimulus—the combined stress of ether and cardiac puncture. We monitored plasma levels of corticosterone both before and after exposure to this potent physiological stressor.

Previous data have demonstrated that pituitary-adrenal responsiveness is affected by nutritional status. In particular, basal levels of plasma corticoids appear to be elevated in humans with protein-calorie malnutrition [3,34] as well as in malnourished animals which have received some preweaning handling [45]. Despite these differences in basal levels, however, malnourished animals appear capable of showing a normal stress response [45]. In order to test for changes in pituitary-adrenal responsiveness, and to be able to separate effects due to iron deficiency from those due to undernutrition, a weight control group was included in this experiment as it was in Experiment 4.

METHOD

Animals

Ten males and 10 females from the same 16 litters which supplied animals for Experiment 4 were randomly selected at 30 days of age from each of the three diet conditions.

Procedure

All blood sampling took place between 0900 and 1100 hr in an anteroom adjacent to the colony room. To obtain basal samples, animals were removed from the colony room and quickly anesthetized with ethyl ether; 0.5 cc of blood was collected in heparinized syringes by cardiac puncture. All samples were taken within 1 to 2 min after exposure to ether, which has been demonstrated to be sufficiently rapid to provide reliable estimates of corticosteroid levels at the time of sampling [15]. These samples were then centrifuged at 2000 rpm for 20 min, after which plasma was extracted and frozen until assayed by the fluorometric method of Glick *et al.* [22].

Etherization+collection of the basal sample served as the stressor in this experiment. Thirty min following the basal sample a second blood sample was collected to determine stress levels of plasma corticosterone. Data were analyzed with a 3(Diet)×2(Sex)×2(Base, Stress) analysis of variance, with the last factor treated as a repeated measure. Post hoc testing was by tests for simple main effects and Newman-Keuls test [47].

RESULTS AND DISCUSSION

The Diet×Time Point interaction was highly significant, $F(2,48)=11.1, p<0.001$ (Fig. 4). Basal corticoid levels of iron deficient animals were similar to those of weight controls, and both of these groups were significantly elevated over ad lib controls ($ps<0.01$). Analysis of the stress response indicated that weight controls showed significantly greater corticoid elevations than ad lib controls which in turn showed greater elevations than iron deficient animals ($ps<0.01$). Thus weight controls showed the highest level of plasma corticosterone following stress while iron deficient animals showed the lowest levels. However, in light of the differences in basal levels it seems appropriate to compare stress increments rather than absolute levels of corticoids. An analysis of stress increments revealed that iron deficient animals showed a significantly smaller increment than both ad lib controls and weight controls ($ps<0.01$), and that these latter two groups did not differ from each other.

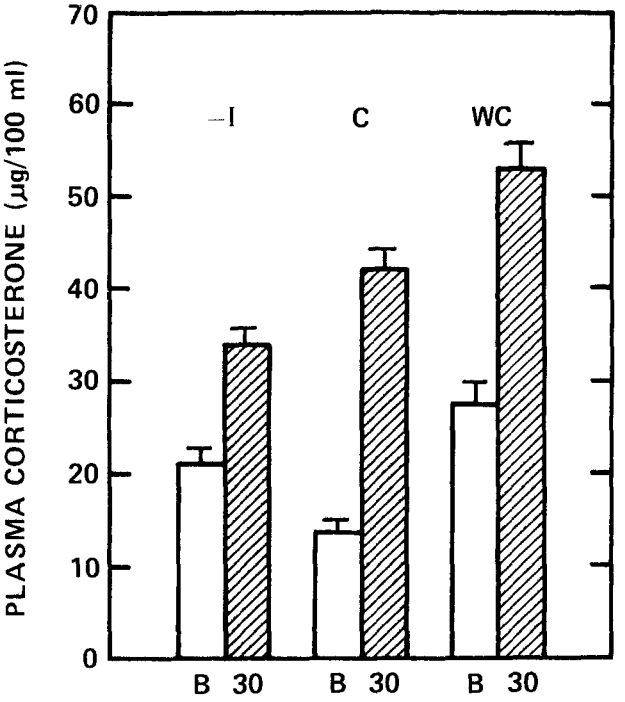

FIG. 4. Plasma levels of corticosterone in iron deficient (-I), ad lib control (C) and weight control (WC) animals. Basal levels (B) and levels measured 30 min after ether and caridac puncture (30) are shown. (Mean+SEM).

There are several interesting findings in this experiment. First we observed that both iron deficient and weight control animals displayed elevated basal levels of plasma corticosterone, a finding that is in accord with the elevated basal levels previously reported in malnourished weanling rats [2,45]. It was previously demonstrated that such elevated levels are due not only to the animal's nutritional status but also to the amount of preweaning handling it has experienced [45]. Thus malnourished animals which have been weighed and thus handled on Days 2, 9 and 16 of age display elevated basal levels of corticoids when compared both with controls which have received similar handling, and with nonhandled malnourished animals. Since both iron deficient and weight control animals in this experiment were subjected to a dietary regimen which reduced their rate of growth, and in addition were handled, i.e. subjected to cage cleaning on Days 10, 14 and 18 of age, the elevated basal levels of corticoids in these animals are consistent with previous findings obtained in severely malnourished animals, and indicate that this effect was not specific to iron deficiency.

Second, we observed that iron deficient animals exhibited a significantly smaller stress increment than either control or weight control animals. Thus although weight control animals had elevated basal levels of corticoids, they displayed a stress increment which was similar to that of controls. This is in accord with results in protein-calorie mal-

nutrition; that is, although malnourished animals display elevated basal levels of corticoids, they appear to be capable of a stress response equivalent to that of controls [45]. This was not true for iron deficient animals. The reduced stress increment seen in the iron deficient group in response to a potent physiological stressor was in marked contrast to the increased responsiveness observed following shock (Experiment 4). This reduced physiological response was in keeping with the reduced behavioral responsiveness observed in novel test situations. The response to shock may be more related to an altered sensitivity to painful stimuli.

GENERAL DISCUSSION

In these experiments we replicated the findings of Dallman *et al.* [14] that an iron deficient diet produces animals which have decreased brain non-heme iron in addition to iron deficiency by a variety of other measures. Iron deficiency also resulted in a somewhat slower weight gain, but our conditions were selected to keep this deficit slight compared to most studies involving malnutrition in the rat. We found that initiating the diet regimen at 18 days gestation or on the day of birth produced more severe iron deficiency with respect to hematocrit and brain non-heme iron in the offspring than providing the diet to lactating females when offspring are 10 days of age. It was also found that brain iron compounds were not affected to a uniform degree when the iron deficient diet regimen was started on the day of birth. Brain non-heme iron (a large category of iron compounds) was decreased by 25 to 35% whereas cytochrome c (a mitochondrial heme-iron enzyme required for the oxidative production of cellular energy) was not decreased at all.

During subsequent behavioral testing we found that iron deficient animals were less responsive than controls in a mildly aversive novel situation (the open field), and ambulated less in an exploratory task (the hole-board). We also observed that iron deficient males exhibited longer response latencies when first exposed to the passive avoidance platform prior to receiving shock. In addition, measurement of plasma levels of corticosterone indicated that although iron deficient animals had elevated basal levels of corticoids, they exhibited a smaller stress increment than controls when exposed to the combined stress of ether and cardiac puncture. Data from the three behavioral tasks taken together with the pituitary-adrenal response to ether and cardiac puncture suggest that iron deficiency may reduce an animal's general responsiveness to environmental stimuli.

Furthermore these data demonstrate the value of including a weight control condition in studies of this type. Because an iron deficient diet provided during early development may restrict weight gain, it becomes important to separate effects due to iron deficiency from those due to undernutrition or to some combination of these two factors. The inclusion of a weight control condition allows some of these effects to be parceled out.

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